NTATION PAGE WR-157-93

Form Approved
OMB No. 0704-0188

ORT DATE	3. REPORT TYPE AND DATES COVERED

4. TITLE AND SUBTITLE
A rapid and sensitive PCR strategy employed for amplification and sequencing of por A from a single colony-forming unit of Neisseria meningitidis.

5. FUNDING NUMBERS

6. AUTHOR(S)
Nancy B. Saunders, Wendell D. Zollinger, and Venigalla B. Rao

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Walter Reed Army Institute of Research
Washington, DC. 20307-5100

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research & Development Command
Ft. Detrick, Frederick, MD. 21702-5012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved For Public Release:
Distribution Umlimited

12b. DISTRIBUTION CODE

13. ABSTRACT *(Maximum 200 words)*

The predicted amino acid sequence was determined for the class-1 outer membrane protein, PorA, from a B:15:P1.7,3 strain of *Neisseria meningitidis* that is currently causing an epidemic of meningitis in Northern Chile. The P1.7,3 PorA showed a unique sequence in the exposed loop 4 of the putative porin structure that is different from all the reported PorA sequences. Based on the nucleotide (nt) sequence of the P1.7,3 *porA*, we designed two sets of PCR (polymerase chain reaction) primers that specifically amplified *porA* from any *N. meningitidis* strain, and a third set of primers that amplified *porA* only from the P1.7,3 strain. Using these primers, we developed a sensitive double hot-start nested PCR (HNPCR) strategy that could amplify *porA* and generate nt sequence from as low as a single colony-forming unit. This strategy consisted of three phases of PCR. The first two phases were designed to generate amplified target DNA that could be directly visualized by ethidium bromide staining starting from one to two molecules of *Neisseria* genome. The third phase was designed to generate a sequence of several hundred nt directly from the amplified DNA. A number of culture-negative cerebrospinal fluid samples from individuals suspected of meningitis during a vaccine trial were analyzed by this strategy to obtain more accurate information on the actual number of cases that occurred in the study and the non-study populations. The basic HNPCR strategy described here could be applied to amplify and sequence target DNAs from any low-copy-number biological sample.

14. SUBJECT TERMS
(Meningococcus; class 1 porin; cerebrospinal fluid; automated sequencing; single-copy amplification; vaccines; clinical diagnosis)

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT

Gene, 137 (1993) 153–162

GENE 07562

A rapid and sensitive PCR strategy employed for amplification and sequencing of *porA* from a single colony-forming unit of *Neisseria meningitidis*

(Meningococcus; class 1 porin; cerebrospinal fluid; automated sequencing; single-copy amplification; vaccines; clinical diagnosis)

Nancy B. Saunders[a,b], Wendell D. Zollinger[b] and Venigalla B. Rao[a]

[a]*Department of Biology, Institute for Biomolecular Studies, The Catholic University of America, Washington, DC 20064, USA; and* [b]*Department Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC 20307, USA. Tel. (1-202) 319-5271*

Received by R.E. Yasbin: 27 April 1993; Revised/Accepted: 23 July 1993; Received at publishers: 3 September 1993

SUMMARY

The predicted amino acid sequence was determined for the class-1 outer membrane protein, PorA, from a B:15:P1.7,3 strain of *Neisseria meningitidis* that is currently causing an epidemic of meningitis in Northern Chile. The P1.7,3 PorA showed a unique sequence in the exposed loop 4 of the putative porin structure that is different from all the reported PorA sequences. Based on the nucleotide (nt) sequence of the P1.7,3 *porA*, we designed two sets of PCR (polymerase chain reaction) primers that specifically amplified *porA* from any *N. meningitidis* strain, and a third set of primers that amplified *porA* only from the P1.7,3 strain. Using these primers, we developed a sensitive double hot-start nested PCR (HNPCR) strategy that could amplify *porA* and generate nt sequence from as low as a single colony-forming unit. This strategy consisted of three phases of PCR. The first two phases were designed to generate amplified target DNA that could be directly visualized by ethidium bromide staining starting from one to two molecules of *Neisseria* genome. The third phase was designed to generate a sequence of several hundred nt directly from the amplified DNA. A number of culture-negative cerebrospinal fluid samples from individuals suspected of meningitis during a vaccine trial were analyzed by this strategy to obtain more accurate information on the actual number of cases that occurred in the study and the non-study populations. The basic HNPCR strategy described here could be applied to amplify and sequence target DNAs from any low-copy-number biological sample.

INTRODUCTION

The causative agent of meningococcal meningitis is *Neisseria meningitidis*, an aerobic Gram$^-$ diplococcus. The species is divided into serogroups that are defined on the basis of the capsular polysaccharide. A total of twelve serogroups are currently recognized, but most systemic disease is caused by serogroups A, B and C. Effective vaccines based on the capsular polysaccharides are available for groups A, C, Y and W135, but vaccine development for group B is still underway. Most group-B vaccines that are under evaluation are based on the prin-

Correspondence to: Dr. V.B. Rao, Department of Biology, 103 McCort Ward Hall, The Catholic University of America, 620 Michigan Ave., N.E., Washington, DC 20064, USA. Tel. (1-202) 319-5271; Fax (1-202) 319-5721.

Abbreviations: *A*, absorbance (1 cm); aa, amino acid(s); bp, base pair(s); cfu, colony-forming unit(s); CSF, cerebrospinal fluid; EtdBr, ethidium bromide; GCG, Genetics Computer Group (Madison, WI, USA); HNPCR, double hot-start nested PCR; kb, kilobase(s) or 1000 bp; mAb, monoclonal antibody(ies); *N.*, *Neisseria*; nt, nucleotide(s); OMP, outer membrane protein; PCR, polymerase chain reaction; PorA, class-1 outer membrane protein of *N. meningitidis*; *porA*, gene encoding PorA; PDS, PCR-directed DNA sequencing; T_m, melting temperature.

cipal outer membrane proteins (OMPs), since the group-B capsular polysaccharide has the same structure as the oligosaccharides present on certain human cells and is therefore a poor immunogen. Meningococci, including serogroup B strains, are further subdivided into serotypes, subtypes, and immunotypes on the basis of the antigenic specificity of two major OMPs and the lipopolysaccharide. The serotype is based on the class-2 or class-3 OMP (a given strain has one or the other but not both) which is also referred to as PorB, and the subtype is based on the class-1 OMP which is called PorA. About 15 different serotypes, twelve subtypes, and eight immunotypes have been identified among group-B strains (Frasch et al., 1985; Zollinger, 1991).

Recent epidemics of group-B disease have occurred in Norway, Cuba, Chile, and Brazil. In Northern Chile, the epidemic of group-B meningococcal disease has been in progress since 1983 with an incidence ranging from ten to 30 cases per 100000 per year. Over 90% of the case isolates from this epidemic have been serotype 15 and subtype P1.7,3 (Cruz et al., 1990). During the period from 1987 to 1989, a vaccine efficacy trial was conducted in the city of Iquique with a group-B vaccine based on the major OMPs. During this study (Zollinger et al., 1991), as efforts were made to identify every possible case of meningitis, several problems associated with accurate, definitive diagnosis of meningococcal disease became evident. A relatively large number of cases accumulated that were clinically suspicious but were culture-negative, and could not be confirmed as meningitis cases or proven to be non-cases with existing methods.

Since mortality among untreated cases of meningitis is high, immediate antibiotic therapy, often prior to collecting a cerebrospinal fluid (CSF) sample, is initiated to stem the rapid progression of the disease. As a result, the number of *N. meningitidis* organisms in clinical samples is often very low. Currently used clinical methods for diagnosis of meningococcal disease such as Gram staining, detection of capsular polysaccharide by latex agglutination, Staph-A co-agglutination, enzyme immunoassay, or counter immuno-electrophoresis, are not sensitive enough to detect the presence of *N. meningitidis* in many of these samples (Zollinger and Boslego, 1992).

The aim of this study was to use PCR as a basis for the development of a highly sensitive and reliable confirmatory diagnostic test for meningococcal disease. Although PCR has been extensively used to rapidly amplify a target DNA by 10^6–10^9-fold, most of the standard PCR experiments require a starting DNA copy number of 10^3–10^5 per sample (Mullis and Faloona, 1987; Arnheim and Erlich, 1992). When the copy number of target genome is low, particularly when it is less than ten copies per sample, inconsistent results were obtained. In most published reports, it was necessary to resort to Southern hybridization of the amplified DNA with a specific ^{32}P-labeled probe in order to determine whether or not the target DNA was amplified (Li et al., 1991; Varas et al., 1991). Except for one report (Mullis, 1991), the problems associated with the amplification and sequencing from one or few molecules of DNA have not been carefully investigated, although such an analysis will have far-reaching applications in many fields of biology. In this paper, we addressed the question of why it is problematic to amplify target DNA from a low-copy-number sample, and developed a double hot-start nested PCR (HNPCR) strategy that could overcome the problems, and not only amplify DNA but also generate DNA sequence directly from as low as a single colony forming unit (cfu).

Although other investigators have used PCR to amplify unique meningococcal DNA sequences from cells in CSF (Kristiansen et al., 1991; Ni et al., 1992), we selected the *porA* gene of *N. meningitidis* as the target gene for developing the PCR strategy because it was thought to be a species-specific gene, and its product, the class-1 OMP, appears to play an important role in human immunity to group-B disease (Zollinger, 1992). We show that the HNPCR strategy can be successfully used to generate nt sequence of *porA* from frozen CSF samples that were not only culture-negative, but were also negative for *porA* amplification by the standard PCR protocols. We believe that this strategy would serve as a general technique to amplify and sequence any target DNA, infectious or otherwise, from a low-copy-number sample.

RESULTS AND DISCUSSION

(a) Selection of *porA* target

The deduced aa sequence and the predicted structure of the class-1 OMP of *N. meningitidis* strain 8529 are shown in Fig. 1. This strain is the prototype strain for subtype P1.3 and is representative of the strains causing an ongoing epidemic in Northern Chile. The P1.3 subtype was first identified and characterized on strains isolated from the Chilean epidemic and has only infrequently been isolated in other countries. Since we were using this strain as a vaccine strain, and the class-1 protein was considered an important component of the vaccine, we wanted to fully characterize this protein and be able to genetically manipulate it for purposes of vaccine production and evaluation. Comparison of this sequence with other published *porA* sequences (Barlow et al., 1989; Maiden et al., 1991; Van der Ley et al., 1991) resulted in the prediction that the variable region of loop 1 contained the epitope for subtype P1.7 [this epitope has recently been described

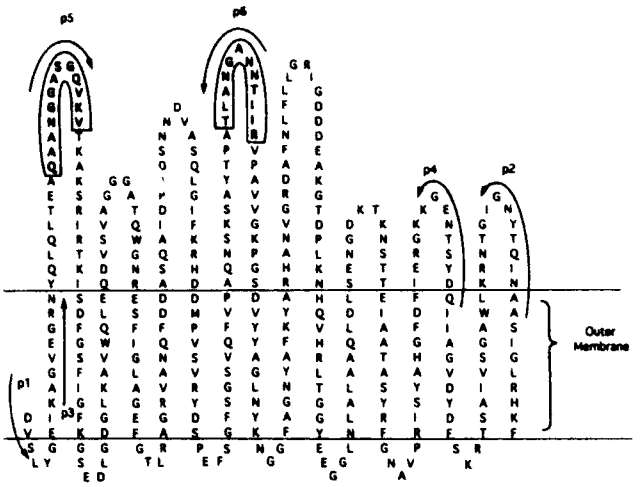


Fig. 1. Deduced aa sequence of PorA P1.7,3 protein arranged to fit into the simplified unrolled β-barrel structural model for porins. Bold letters within the boxes represent the aa that are unique to the P1.7,3 epidemic strain. The regions that correspond to the nt sequence of primers used for PCR and DNA sequencing are marked with arrows. The aa sequence was deduced from the nt sequence of *porA* P1.7,3 of the epidemic strain. To determine the nt sequence of *porA* P1.7,3, two sets of primers (p*B* and p*C*, p*A* and p*D*, Table I) were synthesized from the published sequence of *N. meningitidis* subtype P1.16 *porA* (Barlow et al., 1989). These primer sequences are not specific to the P1.16 subtype, but represent sequences that are conserved among the *porA* subtypes. A unique 1.2-kb fragment was amplified using p*B* and p*C*; p*C* allowed amplification of *porA* beyond the stop codon thereby generating the complete sequence of P1.7,3 *porA* at the 3'-end. For the 5'-end, however, we chose to use p*B* that is part of the sequence coding for the signal peptide portion of PorA P1.16 because a primer upstream from the 5'-end was not highly specific in generating a unique product. Amplification with p*B* and p*C* therefore resulted in the generation of a unique product that codes for the complete mature P1.7,3 PorA. The DNA amplified in two independent PCRs was cloned into M13mp18 and M13mp19 vectors, and was sequenced using Sequenase version-2.0 (US Biochemical, Cleveland, OH, USA). The *porA* sequence in loops 1, 4 and 5 (see below) was reconfirmed by comparing it with that generated by direct sequencing of the PCR-amplified DNA. The sequence was entered into GenBank, and can be accessed using accession No. L02929. The aa sequence was deduced from this nt sequence, and was fit into a simplified β-barrel model of porin structure with the aid of the GCG program (Devereux et al., 1984). The primary criteria used in fitting the sequence to this simplified porin structural model were threefold: (*i*) placing conserved sequences with alternating hydrophilic-hydrophobic residues within the membrane; (*ii*) requiring short turns within the cytoplasm; and (*iii*) placing variable, hydrophilic sequences purported to be antigenic in extended loops on the outside surface. The presence of Tyr and Phe residues aligned along the upper and lower boundaries of the membrane as was found in the X-ray crystallographic analyses of the porin of *Rhodobacter capsulatus* (Weiss et al., 1991) and the porins OmpF and PhoE of *E. coli* (Cowan et al., 1992) further supported the model shown in the figure. Consistent with the published data, the regions of the P1.7,3 PorA that showed clear differences from the other PorA types were found at the tips of the exposed loops 1 and 4 as shown in bold face within the boxes in the figure. The sources of mAb used for epitope mapping studies are as follows. The mAb 12-1-P1.3 was produced in this laboratory by procedures previously described (Zollinger et al., 1984). The mAb were produced using spleen cells from mice immunized with whole organisms or with outer membrane vesicles. The mAb specific for P1.7 (MN14C11.6) was provided by Dr. Jan T. Poolman, Bilthoven, The Netherlands.

as a 'masked' P1.7 due to a specific 9-bp deletion downstream from this region (Wedege et al., 1993)], and the variable region of loop 4 contained the epitope for subtype P1.3 (the unique aa are shown in bold letters in Fig. 1). Peptides corresponding to the unique regions were synthesized, and were tested for binding to P1.7- or P1.3-specific monoclonal antibodies by ELISA and by Geysen pin techniques (Geysen et al., 1984). The P1.7-specific mAb specifically bound to the loop-1 peptide (aa 18–40) at a serum dilution of 1:204 800, but did not bind to either the loop-4 peptide (aa 170–192) or the outer membrane complex in which the epitope is masked. We confirmed the P1.7-specific epitope to the peptide sequence SGQ (aa 32–34) by Geysen pin analysis (McGuinness et al., 1990). Similarly, the P1.3-specific mAb specifically bound to the loop-4 peptide at a serum dilution of 1:102 400 and also to outer membrane complex at a serum dilution of 1:819 200, but not to the loop-1 peptide. The P1.3-specific epitope was further mapped to the peptide sequence ANGANNTI (aa 177–185) by Geysen pin analysis. These direct binding studies clearly identified the P1.7- and P1.3-specific epitopes in loops 1 and 4, and are consistent with the above predictions that are based on nt sequence analysis.

(b) Design of primers for specific amplification of *porA*

To identify unique regions in *porA*, we first compared the *porA* subtype P1.7,3 sequence determined in this study with the published sequences of *porA* from other subtypes, and also that of *porB*. Based on these comparisons, two sets of primers that are complementary to the termini of *porA* were chosen for PCR (Table I). These primers were then tested for their specificity using a number of strains of *Neisseria* species and *Branhamella catarrhalis* that often exist as normal flora in throat cultures. *Escherichia coli* and *Haemophilus influenzae*, both of which are known to cause meningitis, were also included in these experiments. The outermost primers p1 and p2, as shown in Fig. 2A, were found to yield a highly specific 1.1-kb product only with *N. meningitidis* P1.7,3 (lane 3) and other subtypes of *N. meningitidis* (Fig. 2B and data not shown), but not with either the commensal *Neisseria* strains (lanes 4–9), or with *H. influenzae* or *E. coli* (lanes 1 and 2). Similarly, the inner nested primers p3 and p4 also specifically amplified a 1-kb *porA* product only from the *N. meningitidis* strains (lanes 3–9, Fig. 2B), but not with the commensal strains (lanes 10–13, Fig. 2B, and data not shown). It should be mentioned that another nested primer, covering nt 970–995, was less specific as it occasionally amplified *porA* from commensal strains (data not shown). This was probably because this primer corresponded to an intramembrane region that is conserved among *Neisseria* porins.

TABLE I

Primers used in PCR and direct sequencing

Primer sequence[a]		Description[b]	Nucleotide location on P1.3 DNA sequence[c]
Cloning primers			
p*A*	CGT ATC GGG TGT TTG CC	Upstream	
p*B*	GCC CTC GTA TTG TCC GCA	Leader	19–35
p*C*	GGG CTG AAG CAG ATT GG	Downstream	
p*D*	GCC GAT GCC GGT ATT GCG	Inner reverse	1080–1063
Amplification primers			
p1	GCG GCC GTT GCC GAT GTC AGC C	Outer forward	21–42
p2	GCG GCA TTA ATT TGA GTG TAG TTG CC	Outer reverse	1124–1098
p3	CAA AGC CGG CGT GGA AG	Inner forward	55–72
p4	GAT CGT AGC TGG TAT TTT CGC C	Inner reverse	1011–990
p5	GTG GAG CGA GCG GTC AG	P1.7-specific forward	118–134
p6	ATT AGC ACC ATT AGC AAG AG	P1.7-specific reverse	574–556
Sequencing primers			
pI	CAC TCC CCT TAA AGC CGA T	Downstream loop 1	213–195
pII	CTC AGA CCG GCA TAA TAC A	Downstream loop 4	641–624
pIII	CCC ACA TTG GCG TGT CTC GC	Downstream loop 4	706–687

[a] Primer sequences p*A*–p*D* are from the *porA* P1.16 sequence of Barlow et al. (1989). Primer sequences p1–p6 and pI–pIII are from the *porA* sequence of 8529, the prototype P1.3 strain (this work).

[b] Positions of primers p*A*–p*D* refer to the locations of these primers relative to the expressed class 1 porin protein. Positions of primers p1–p6 are shown with arrows in Fig. 1. Positions of primers pI–pIII are from sequences that flank the exposed variable loops 1 and 4.

[c] The numbers correspond to the exact positions of the nucleotides on the *porA* P1.7,3 sequence. The entire *porA* P1.7,3 sequence was entered into GenBank and can be accessed with accession No. L02929.

A third set of nested primers, p5 and p6, were then designed which were specific for *porA*, subtype P1.7,3. These subtype-specific primers were required for identification of the epidemic subtype of meningococci in Iquique, Chile. These primers were designed in such a way that they are complementary to the unique variable regions in loop 1 and loop 4 of the epidemic strain (see Fig. 1). To test the specificity of these primers, the 1.1-kb *porA* products amplified from eight well-characterized subtypes and two non-subtypable strains were used for a second amplification by the subtype-specific innermost primers p5 and p6 (Fig 2C). A unique 500-bp product could be amplified only from the type P1.7,3 template (lane 4), whereas the *porA* of other subtypes did not serve as a target for amplification by these primers (lanes 1–3 and lanes 5–10).

It should be pointed out that all the above PCR experiments were done using an excess of template DNA (approx. 10^6 copies of genome per reaction) in order to rigorously establish the specificity of the primers. The copy number used in these experiments is far greater than what one would expect to encounter in a clinical sample.

(c) A PCR strategy to amplify *porA* from a single cfu of *N. meningitidis*

One of the challenging problems in the diagnosis of meningococcal meningitis is the detection of *N. meningitidis* in clinical samples from suspicious cases that are culture-negative, and therefore contain a very low copy number of target DNA. We sought a sensitive PCR strategy that would have the following features, and would be able to definitively establish the status of every suspicious case of meningitis: (*i*) it should amplify *porA* from the theoretical limit of one cfu of *N. meningitidis* per sample, (*ii*) the amplified DNA should be visible as a unique EtdBr-stained DNA band upon agarose gel electrophoresis; (*iii*) it should amplify *porA* from a crude clinical sample such as a CSF of an infected individual, and (*iv*) it should be compatible with the rapid PDS strategy that we developed recently (Rao and Saunders, 1992), to generate the nt sequence of the important variable regions.

Our main focus was to address the first two objectives since reports on amplification of target DNA from less than ten copies of the genome often failed to generate a unique product (Varas et al., 1991). The amplified DNA usually migrated as a smear. Consequently, Southern blotting and hybridization with a specific probe were required to confirm the presence of target DNA. Indeed, many of our initial attempts to amplify *porA* from the CSF of patients suspected of having meningococcal infections led to the appearance of a smear rather than a unique product (data not shown). We hypothesized that the main contributing factor for the DNA smear is mis-

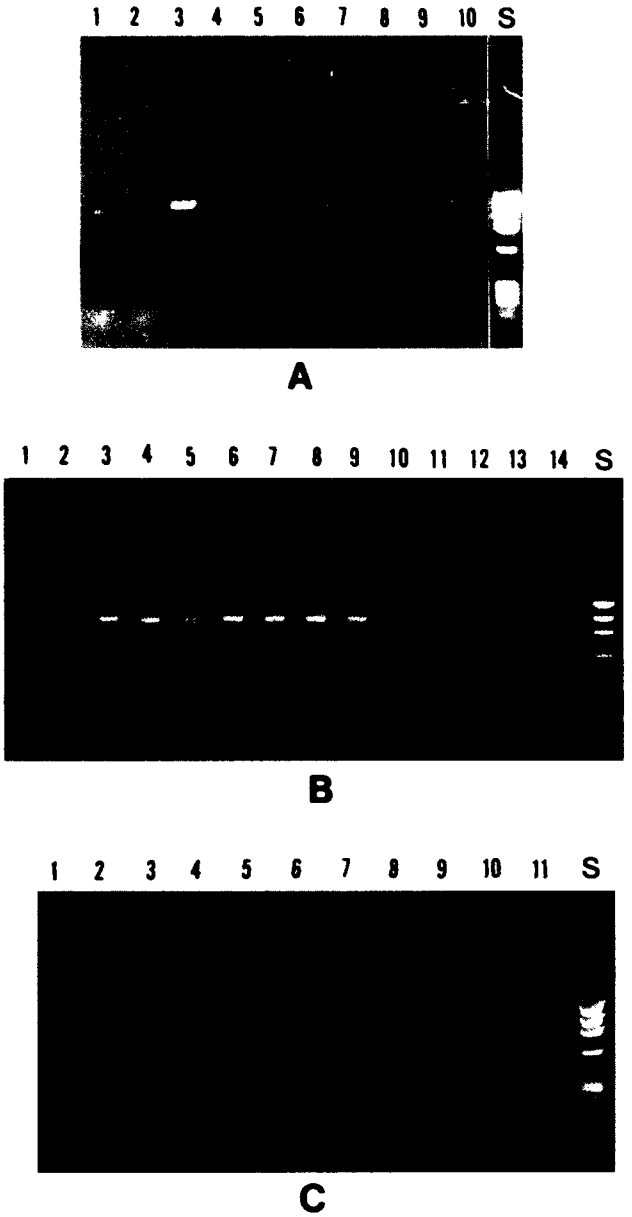


Fig. 2. Specific amplification of *porA* from *N. meningitidis* by three sets of primers. The sources of strains used in these experiments are as follows. *N. meningitidis* strains 8529 (prototype of subtype P1.3), 99M, and 1901 were from the culture collection of the Walter Reed Army Institute of Research. Prototype strains M992, M982, S3032, M981, M136, and S3446 were obtained from Dr. Carl Frasch, Bethesda, MD; strain H355 was obtained from Dr. L. Oddvar Froholm, Oslo, Norway. The commensal *Neisseria*, *B. catarrhalis* and *E. coli* strains were from the ATCC, Rockville, MD. The *H. influenzae* strain was provided by Dr. Arthur Branstrom, Washington, DC. The primers required for PCRs were synthesized using an ABI Model-391 PCR-mate oligodeoxynucleotide synthesizer, and were purified by gel filtration using PD-10 columns (Pharmacia-LKB) (Table I). An isolated colony of *N. meningitidis* or other appropriate strain was picked and resuspended in 1.0 ml Gey's balanced salt solution containing 0.2% gelatin. An aliquot of this suspension, containing approximately 10^6 organisms, was heated at 95°C for 10 min, cooled quickly on ice, and the PCR components (200 μM of each dNTP/50 pmol of each primer/2.5 units of *Taq* polymerase) were added directly to this crude sample to a final volume of 100 μl per reaction. PCRs were done in a Perkin-Elmer thermal cycler (N801-0150) for 30 cycles. Each cycle consisted of denaturation of DNA at 95°C for 1 min, annealing for 1 min either at 50°C (in the case of primer sets p3 and p4, and p5 and p6) or 70°C (in the case of primers p1 and p2), and extension at 70°C for 2 min. (Panel **A**) The outermost primers p1 and p2 amplified the 1.1-kb *porA* only from *N. meningitidis*. The bacterial strains used were as follows: lane 1, *H. influenzae*; lane 2, *E. coli*; lane 3, *N. meningitidis*; lane 4, *N. perflava*; lane 5, *N. lactamica*; lane 6, *N. flava*; lane 7, *N. sicca*; lane 8, *N. subflava*; lane 9, *N. flavescens*; lane 10, *B. catarrhalis*. (Panel **B**) The inner nested primers p3 and p4 amplified the 1-kb fragment from all *N. meningitidis* subtypes tested. The bacterial strains used were as follows: lane 1, *E. coli*; lane 2, *H. influenzae*; lane 3, M992 (P1.1); lane 4, 99M (P1.2); lane 5, 8529 (P1.7,3); lane 6, 1901 (P1.6); lane 7, M982 (P1.9); lane 8, H355 (P1.15); lane 9, S3032 (P1.12,16); lane 10, *N. lactamica*; lane 11, *N. polysaccharea*; lane 12, *N. subflava*; lane 13, *B. catarrhalis*; lane 14, reagent blank. (Panel **C**) The innermost nested primers p5 and p6 amplified only the subtype P1.7,3-specific 500-bp fragment starting from an excess of *porA* template from each strain. The subtypes used were as follows: lane 1, M992 (P1.1); lane 2, 99M (P1.2); lane 3, 1901 (P1.6); lane 4, 8529 (P 1.7,3); lane 5, M982 (P1.9); lane 6, H355 (P1.15); lane 7, S3032 (P1.12,16); lane 8, M981 (non-typable); lane 9, M136 (non-typable); lane 10, S3446 (P1.14); lane 11, reagent blank. Lane S represents *Hae*III-digested ϕX174 DNA as size standards; the sizes of the four bands from the top are 1353, 1078, 872 and 603 bp, respectively. The samples were electrophoresed on a 1.2% agarose gel and stained with EtdBr.

priming at non-target sites due to short stretches of homology between the genomic DNA and the 3′-end of the primer (a schematic representation is shown in Fig. 3). Therefore, after the first few cycles of PCR, the target DNA will constitute only a minor component of the amplified DNA, whereas the major fraction is expected to be the misprimed nonspecific DNA thereby generating a DNA smear (Fig. 3). We tested a number of strategies to overcome this problem, and developed a HNPCR strategy to specifically amplify *porA* from one or a few copies of *N. meningitidis* genome (see legend to Fig. 4 for experimental details). The basic premise of these experiments was as follows: even though the amplified DNA after the first phase PCR is predicted to have a mixture of target DNA and non-target DNAs of random lengths, it should be possible to selectively amplify only the target DNA from this heterogenous mixture by performing a second phase PCR using an inner set of nested primers (Fig. 3). Since the target DNA after first phase PCR will be at a sufficiently high copy number, and since the probability of having a sequence that is complementary to the inner nested primers on both ends of the non-target DNA will be infinitesimally small, we expected that the target DNA now should be amplified efficiently to generate a unique product (Fig. 3). In the third and final step, the amplified DNA will be sequenced by asymmetric PCR using the PDS strategy we developed earlier (Rao and Saunders, 1992). A number of variations of this basic approach were tested using defined samples containing 0.25–40 cfu of *N. meningitidis*. The HNPCR strategy consistently generated a unique product that could be visualized simply by

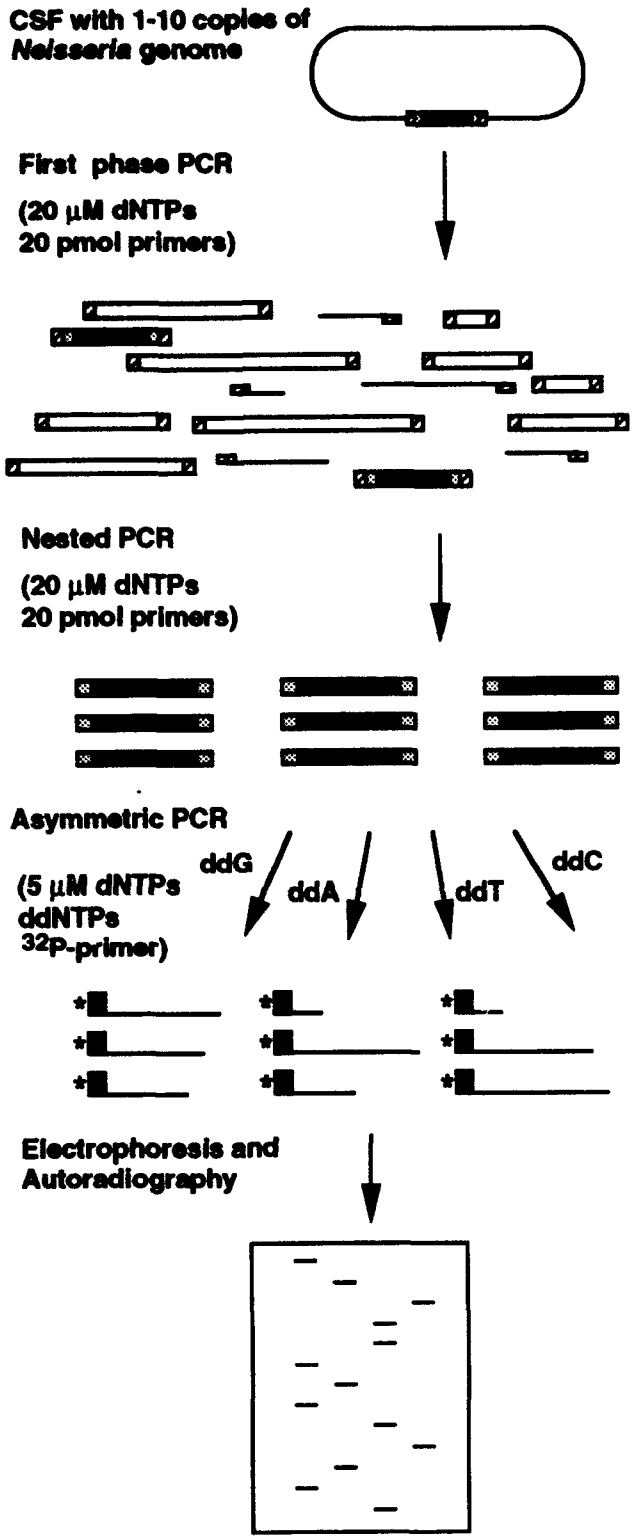


Fig. 3. Schematic representation of the HNPCR strategy to generate nt sequence of target DNA directly from a crude clinical sample containing one to ten copies of *N. meningitidis* genome. The target DNA is represented as a central black box flanked by two small shaded boxes representing regions that correspond to outer and inner nested primers. The PCR products drawn with double lines represent double-stranded DNA, and those with single lines represent single-stranded DNA. The stars in the products of asymmetric PCR represent ^{32}P-label at the 5'-end of the products.

EtdBr staining. Indeed, PCR of serial dilutions of *N. meningitidis* repeatedly showed successful amplification of the unique *porA* product from the theoretical limit of one cfu per sample (Fig. 4). As shown in lanes 5–7, all three samples that had one cfu showed the 1.1-kb size *porA* product, while only one of three samples that had an average of 0.25 cfu or less, showed the product (lanes 8–13). These data therefore demonstrated that the HNPCR strategy is highly sensitive and could amplify *porA* from a single *N. meningitidis* organism.

(d) Amplification of *porA* from CSF samples

The strategy was then tested by amplifying *porA* from the CSF of patients suspected of having meningococcal meningitis. The cases had been rated by physicians as suspicious, moderately suspicious, or highly suspicious, based on the clinical features associated with these patients. These samples were frozen for two years at −70°C, and had given a negative result by the culture and Gram-stain techniques, and also by standard PCR protocols (Arnheim and Erlich, 1992). These samples contained either no meningococci or a minimal number of non-viable organisms, and therefore provided an ideal test of this strategy to analyze *porA* in clinical samples. In order to rigorously test the strategy, these experiments, including the positive and negative controls, were done in a blinded manner. The first set of PCR cycles were done using primers p1 and p2, and the second set was done either with primers p3 and p4 (Fig. 5A) or with the subtype-specific primers p5 and p6 (Fig. 5B). The data in Fig. 5A show that a DNA band of the expected 1-kb size was amplified from one out of three suspicious cases (lanes 3–5), one out of two moderately suspicious cases (lanes 6–7), and two out of three highly suspicious cases (lanes 8–10). When the second set of PCR cycles was done using the subtype-specific primers p5 and p6, all but one of the positive samples (lane 5) showed the unique 500-bp product of the P1.7,3 strain.

(e) Determination of nt sequence of the DNA amplified from CSF samples

In the context of our vaccine efficacy studies, it is important to be able to determine the nt sequence in the variable regions of *porA* directly from the PCR products. This would allow us to quickly confirm the PCR data, determine the sequence of variable regions that were different from the P1.7,3 prototype strain, and detect any microheterogeneity in sequence that could affect recognition of PorA by vaccine-induced antibodies. An aliquot of the PCR mixture following the second phase PCR, without any purification of template, was directly used for sequencing by a third phase asymmetric PCR in the presence of a single ^{32}P-labeled primer (Rao and

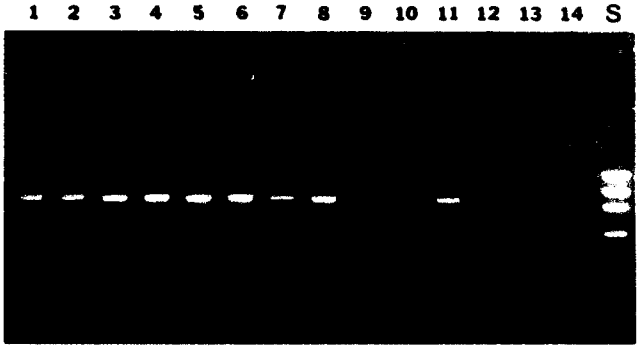


Fig. 4. Amplification of *porA* from the theoretical limit of one cfu of *N. meningitidis*. Lanes 1 and 2: 40 cfu; lanes 3 and 4: 5 cfu; lanes 5, 6 and 7: 1 cfu; lanes 8, 9 and 10: 0.25 cfu; lanes 11, 12 and 13: <0.25 cfu; lane 14: reagent blank; lane S represents *Hae*III-digested ϕX174 DNA as size standards. **Methods:** A log phase broth culture of *N. meningitidis* (A_{650} = 0.35) was serially diluted in Gey's balanced salt solution to give a series of samples containing approximately 40–0.25 organisms per 10 µl. The diluted sample was heat-denatured and used directly for amplification. A duplicate 10 µl sample was spread on a gonococcal agar plate with defined supplements (Schneider et al., 1988) to determine the number of cfu. To rule out any nonspecific clumping of the organism, the samples at different dilutions were dried on slides, Gram-stained, and examined microscopically. No clumping was observed in any of the cultures tested. To the above sample, 25 µl H_2O and a drop of oil were added. The cells were heat-denatured at 95 C for 10 min. To the denatured sample, 10 µl of 10 × *Tub* polymerase buffer as supplied by the vendor (Amersham)/20 µM of each dNTP/20 pmol of each primers p1 and p2, and H_2O to 90 µl, were added. Hot-start PCR was done by incubating the samples in the thermal cycler at 90 C for 5 min and then adding 10 µl of diluted *Tub* polymerase (2.5 units) (Amersham). In the initial experiments, the sensitivity of the PCR strategy was tested using either *Taq* polymerase (2.5 units) (Perkin-Elmer) or *Tub* polymerase (2.5 units). *Tub* polymerase, for unknown reasons, consistently showed superior amplification when compared to *Taq* polymerase. Subsequent experiments were therefore performed only with *Tub* polymerase. *PorA* was amplified for 30 cycles using a rapid two-step protocol consisting of denaturation of DNA at 95 C for 1.5 min, and annealing and extension at 70 C for 3 min with a 2 s extension per cycle. The second phase nested PCR was done by directly adding a 10 µl aliquot of the first phase PCR mixture to the second phase reaction components. The composition of the reaction mixture and conditions for hot-start for the nested PCR were exactly the same as the first phase PCR except that the primers p1 and p2 were replaced with either p3 and p4, or p5 and p6 (Table I). The cycling conditions for second phase PCR are as follows: the first three cycles consisted of denaturation at 95 C for 3 min, annealing at 50 C for 2 min and extension at 72 C for 2 min; this was followed by 27 cycles, each of which consisted of denaturation at 95 C for 1 min, annealing at 50 C for 1 min and extension at 72 C for 1 min. A 5 µl sample of the PCR mixture was analyzed by electrophoresis on a 1.2% agarose gel. For successful amplification from a single cfu, it was important to test every new batch of reaction components by the serial dilution technique described above to ascertain the high sensitivity of the strategy. The reagents were then aliquoted and kept frozen for future experiments to maintain the same degree of sensitivity. Since the strategy is extremely sensitive, care was taken to prevent any external contamination. This was particularly important during the transfer of samples from the first phase PCR to the second phase PCR. This was always done in an isolated area while taking a number of precautions recommended in the literature (Kwok and Higuchi, 1989). In addition, a number of reagent controls were performed in each experiment to ascertain that the reagents were free of any contamination. The reagents were kept in an isolated area, and were exclusively used for this work.

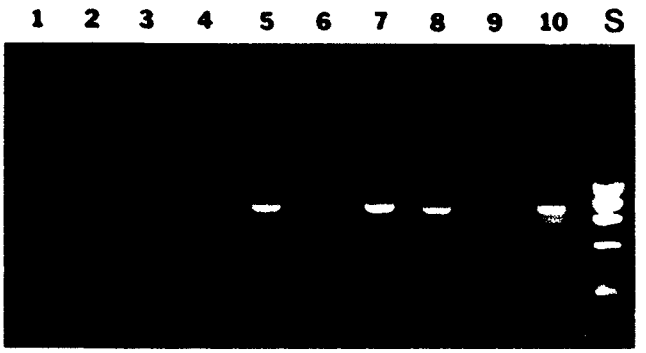
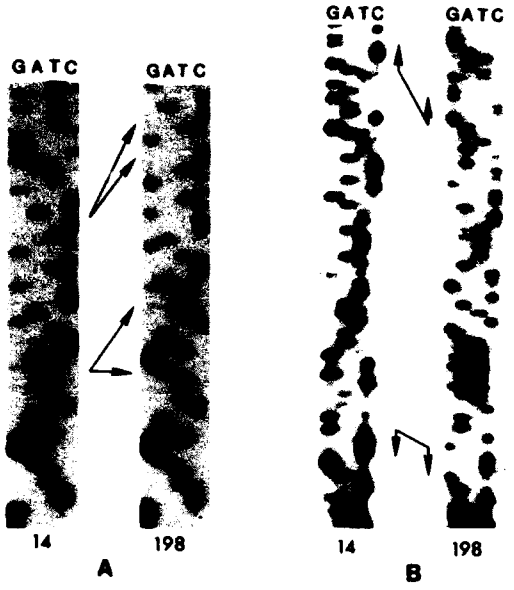


Fig. 5. Identification of meningococcal DNA in unknown frozen CSF samples using the HNPCR strategy. A 100 µl sample of CSF was centrifuged at 10000 rpm for 10 min, and the pellet was resuspended in 25 µl of sterile water, and denatured at 95 C for 10 min. The PCR components were added directly to the sample, and thermal cycling was carried out according to the conditions described above. Each PCR experiment involving CSF samples also included a known positive, a known negative, and a reagent control. The CSF samples including the positive and negative controls were analyzed in a blinded manner and were decoded after completion of the experiment. (Panel **A**) Primers p1 and p2 were used in the first set of PCR cycles and primers p3 and p4 were used in the second set of PCR cycles. (Panel **B**) Primers p1 and p2 were used in the first set of PCR cycles, and primers p5 and p6 were used in the second set of PCR cycles. Lane 1, a known positive CSF sample that showed a high titer of meningococci by culturing; lane 2, a known negative; lanes 3–5, suspicious cases; lanes 6 and 7, moderately suspicious cases; lanes 8–10, highly suspicious cases; lane S represents *Hae*III-digested ϕX174 DNA as size standards. The samples were electrophoresed on a 1.2% agarose gel and stained with EtdBr.

Saunders, 1992). The sequence ladders of the variable regions of *porA* amplified from the CSFs are shown in Fig. 6. Amplified products that were PCR positive for subtype P1.7,3 (as shown in Fig. 5B), as expected, gave a sequence that matched perfectly with both loop 1 and loop 4 sequences shown in Fig. 1 (panels of Case 14 in Fig. 6). The single P1.7,3-negative case in the above experiment (lane 5, Fig. 5B) was also confirmed to be negative by DNA sequencing (panels of Case 198 in Fig. 6). The nt sequence of this strain was found to match perfectly with the sequence of variable loops 1 and 4 of strain



PANEL A

Case 14: TAACTT.....TTACCTGACCTCTCGCTCC.....ACC

Case 198: TAACTTTAGTAACTTTTACCTGACCGCTCGCTCCCGCTCCACC

PANEL B

Case 14:
AGCCGGAACGCGAATAATTGTATTATTAGCACCATTAGCAAGAGTAGC

Case 198:
AGCCGCAACTTTTTTAGCTACGCCATTTTCCACCGCCACATA...AGC

Fig. 6. Determination of nt sequence of *porA* amplified from CSF samples. The amplified *porA* was directly sequenced using the PCR-directed sequencing (PDS) strategy described previously (Rao and Saunders, 1992). A 5 µl aliquot of the PCR mixture as in Fig. 5A was mixed with 5 µM each of four dNTPs, 30 pmol ^{32}P-labeled primer, either 100 µM ddGTP, or 750 µM ddATP, or 750 µM ddTTP, or 1 mM ddCTP, 125 µM Mn^{2+} and 2.5 units of *Tub* polymerase. PCR cycling was done according to the conditions described earlier (Rao and Saunders, 1992). The labeled products were resolved by electrophoresis on a sequencing gel containing 6% polyacrylamide and 8 M urea. The gel was dried and autoradiographed by exposing to an X-OMAT AR (Kodak) film. Since the variable loops 1 and 4 are of most interest in determining the type specificity, sequencing was done using primers pI, pII or pIII (Table 1) to cover these regions. (**A**) Sequence ladders were generated using loop-1 primer, pI. Note a 9-nt and a 3-nt insertion (bracketed with arrows) in Case 198 when compared to Case 14. (**B**) Sequence ladders were generated using loop-4 primer, pII. Note that the sequence in between the top and lower arrows is different between Case 14 and Case 198. The actual sequences of the cases are shown at the bottom of the figure.

M1080 that was recently reported by Maiden et al. (1991). This was the only non-P1.7,3 strain that we found among the 70 CSF samples analyzed thus far (data not shown).

(f) Conclusions

(*1*) We determined the nt sequence and the predicted structure of *N. meningitidis* PorA subtype P1.7,3 that is currently causing an epidemic in Chile. The sequence is consistent with the other published sequences (Barlow et al., 1989; Maiden et al., 1991; Van der Ley et al., 1991), but is unique in the variable region of loop 4, which we have shown to contain the subtype P1.3 epitope. Since the P1.7,3 subtype has been responsible for over 90% of the group-B disease in Northern Chile, this sequence information is critical for the development of the HNPCR strategy as well as the development and evaluation of potential vaccines for use in that area.

(*2*) We selected three sets of primers that are highly specific for *N. meningitidis*. Of these, two sets of primers, p1 and p2, p3 and p4, specifically amplified a 1.1-kb and a 1-kb product of *porA*, respectively, whereas the third set of primers, p5 and p6, specifically amplified the 0.5-kb size subtype P1.7,3-specific *porA* product. Some strains of *N. meningitidis* do not express any detectable PorA and are nonsubtypable. Several of these were included in panels testing the primers. All were positive for *porA* and yielded the predicted product with the exception of one isolate which was found to have a deletion of the gene (data not shown). *N. gonorrhoeae*, which appears to harbor a silent *porA* gene, however showed amplification of a 1.1-kb product with the outer primers p1 and p2, and a 0.6-kb product with inner nested primers p3 and p4, suggesting a potentially rearranged *porA* sequence in this organism (data not shown). Barlow et al. (1989) previously suggested the presence of a *porA*-related sequence on the basis of Northern blot analysis. This should not however affect the specificity of the assay or its usefulness in routine testing of clinical samples because the 0.6-kb product generated with p3 and p4 from *N. gonorrhoeae* could be easily distinguished from the 1-kb size *N. meningitidis porA* product, and also *N. gonorrhoeae* is unlikely to be present in the CSF samples. Determination of the subtype specificity of *N. meningitidis* has been very useful from the point of view of vaccine efficacy evaluation. This is true because the vaccine is considered to be primarily a subtype-specific vaccine and may not protect against heterologous subtypes. The subtype-specific primers p5 and p6 could be simply replaced with another pair of subtype-specific primers in a different epidemiological setting, or if multiple subtypes are causing disease, a panel of subtype-specific primers could be used to determine the subtype of the isolates.

(*3*) The problems associated with amplification of DNA from low-copy-number samples have not been fully investigated. From this study, it appears that minimizing the mispriming events, particularly those occurring during the initial cycles of PCR, played a critical role in amplifying a unique DNA fragment from a low-copy-number sample. We believe that mispriming is primarily due to two reasons. First, most protocols recommend the following sequence of steps for standard PCR: (*i*) boiling the complete PCR mixture, (*ii*) cooling the samples imme-

diately on ice, and (*iii*) denaturation of DNA at 94 C to initiate the first PCR cycle (Arnheim and Erlich, 1992). Though these steps were intended to denature any protease or nuclease contamination in the crude sample, it is known to generate extensive mispriming (Mullis, 1991), which will be highly problematic while amplifying target DNA from a low-copy-number sample. This is because, during the cooling step and the subsequent denaturation step of the first PCR cycle when the temperature of the reaction mixture is rising from 4°C to 94°C, the DNA polymerase is active and would extend any template-primer complexes available in the reaction mixture. While the number of specific complexes is one or a few in a low-copy-number sample, there will be numerous nonspecific complexes that are formed as a result of short stretches of complementarity between the template and the 3'-end of the primer that is present in large excess. As a result, the nonspecific extension products predominate in the reaction mixture after the first PCR cycle (Fig. 3). We designed the HNPCR strategy to overcome this problem in two ways: first, the sample was denatured separately in the absence of any PCR components, and was then added to the PCR mixture; secondly, the first cycle of PCR was initiated by a hot-start procedure (D'Aquila et al., 1991) in which the DNA polymerase was added only after heating the samples to 90°C. The second problem in amplifying target DNA from a low-copy-number sample is the mispriming events that occur as a result of low stringency of annealing. This was minimized by designing the primers that have high estimated T_m values, and having the T_m values of the two primers close to each other. This allowed the performance of PCRs under highly stringent conditions with an annealing temperature that is close to the T_m value. In some instances, we even used an annealing temperature that is the same as the estimated T_m value without significantly affecting the efficiency of amplification (data not shown).

Even with the above modifications, we found that it was practically impossible to eliminate the mispriming events altogether. A DNA smear rather than a unique product was obtained after the first phase PCR in most of the experiments. This was because the synthesis of non-target DNAs from misprimed complexes is an inherent problem associated with the way the PCR technique works. In a low-copy-number sample, the ratio of DNA polymerase (10^9 molecules), or primers (3×10^{13} molecules each), to that of target DNA (1–10 molecules) is overwhelmingly high. Therefore, even under the most stringent PCR conditions, there will be a formation of transient unstable complexes between random regions of template and primers, and between primers. These complexes, at a certain frequency, will be converted to productive stable complexes by rapid addition of a few nt at the 3'-end of the primer by the highly active DNA polymerase that has a synthetic rate on the order of about 4000 nt per minute (Arnheim and Erlich, 1992). This problem will be further exacerbated if the test sample contained extraneous nonspecific DNA, which is almost always the case with a crude clinical sample. Indeed, it is known that *Taq* polymerase could prime DNA synthesis based on a two nucleotide homology, or in some instances no homology at all, generating primer dimers, multimers, and secondary recombination products in standard amplification reactions containing a high copy number of target DNA (Arnheim and Erlich, 1992). Reports that recommend extension of PCR cycling to 40–50 cycles to amplify DNA from a low-copy-number sample are counter-productive since this would lead to efficient amplification of nonspecific products that are predominantly present in the PCR mixture rather than the unique product (Mullis, 1991). We found that the only way to generate unique amplified product consistently from a low-copy-number sample is to do a nested PCR in addition to the modifications described above. Nested PCR selectively amplified the target DNA from a pool of target and non-target DNAs since the existence of complementary sequences on both ends of a non-target DNA is practically zero. Some mispriming events will undoubtedly occur in this step, but now since the starting copy number of target DNA is high, these events will be far outweighed by the specific amplification events.

(*4*) The power of this single copy DNA amplification strategy was further enhanced by extending it to generate nt sequence directly from the amplified DNA using a direct sequencing strategy. The composition of PCR mixtures was designed in such a way that the amplified DNA from the nested PCR could be directly used for sequencing without any time consuming template purification procedures. Therefore, using the complete strategy involving three successive phases of PCR, complete sequence of the target DNA was generated starting from one or a few DNA molecules. The sequence data generated from CSF samples of suspicious cases of the Iquique epidemic provided additional reliable data on the incidence of meningococcal meningitis among the volunteers enrolled in the study, and on the effectiveness of the vaccine.

(*5*) We believe that the HNPCR strategy could be applicable to a wide variety of genetic analyses in basic as well as clinical research labs. For example, it could be used for definitive diagnosis of genetic diseases at a very early stage of fetal development. It could also be used for definitive diagnosis of HIV in the initial stages of infection, or when the virus exists in a dormant stage. Because of minimal manual manipulations involved, the complete procedure could be automated for rapid analysis of a large number of samples.

ACKNOWLEDGEMENTS

We gratefully acknowledge Dr. John Boslego, principal investigator of the vaccine field trial in Iquique, Chile. We acknowledge the efforts of Drs. Carlos Cruz, Marcela Martinez, and Ricardo Bustamante in collecting and preserving the CSF samples and completing special case reports on the patients involved in the study. We thank Dr. Susan Garges for critically reading the manuscript. V.B.R. is supported by a grant from the Department of Energy (DE-FG05-90ER60996).

REFERENCES

Arnheim, N. and Erlich, H.: Polymerase chain reaction strategy. Annu. Rev. Biochem. 61 (1992) 131–156.

Barlow, A.K., Heckels, J.E. and Clarke, I.N.: The class 1 outer membrane protein of *Neisseria meningitidis*: gene sequence and structural and immunological similarities to gonococcal porins. Mol. Microbiol. 3 (1989) 131–139.

Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R., Pauptit, R.A., Jansonius, J.N. and Rosenbusch, J.P.: Crystal structures explain functional properties of two *E. coli* porins. Nature 358 (1992) 727–733.

Cruz, C., Pavez, G., Aguilar, E., Garcia, J., Ruiz, S., Canepa, I., Martinez, M., Boslego, J., Zollinger, W.D. and Arthur, J.D.: Serotype specific outbreak of group B meningococcal disease in Iquique, Chile. Epidemiol. Infect. 105 (1990) 119–126.

D'Aquila, R.T., Bechtel, L.J., Videler, J.A., Eron, J.J., Gorczyca, P. and Kaplan, J.C.: Maximizing sensitivity and specificity of PCR by pre-amplification heating. Nucleic Acids Res. 19 (1991) 3749.

Devereux, J., Haeberli, P. and Smithies, O.: A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. 12 (1984) 387–395.

Geysen, H.M., Meloen, R.H. and Barteling, S.J.: Use of peptide synthesis to probe viral antigens for epitopes to a resolution of a single amino acid. Proc. Natl. Acad. Sci. USA 81 (1984) 3998–4001.

Frasch, C.E., Zollinger, W.D. and Poolman, J.T.: Serotype antigens of *Neisseria meningitidis* and a proposed scheme for designation of serotypes. Rev. Infect. Dis. 7 (1985) 504–510.

Kristiansen, B., Ask, E., Jenkins, A., Fermer, C., Radstrom, P. and Skold, O.: Rapid diagnosis of meningococcal meningitis by polymerase chain reaction. Lancet 337 (1991) 1568–1579.

Kwok, S. and Higuchi, R.: Avoiding false positives with PCR. Nature 339 (1989) 237–238.

Li, H., Cui, X. and Arnheim, N.: Direct electrophoretic detection of the allelic state of single DNA molecules in human sperm by using the polymerase chain reaction. Proc. Natl. Acad. Sci. USA 87 (1991) 4580–4584.

Maiden, M.C.J., Suker, J., McKenna, A.J., Bygraves, J.A. and Feavers, I.M.: Comparison of the class 1 outer membrane proteins of eight serological reference strains of *Neisseria meningitidis*. Mol. Microbiol. 5 (1991) 727–736.

McGuinness, B., Barlow, A.K., Clarke, I.N., Farley, J.E., Anilionis, A., Poolman, J.T. and Heckels, J.E.: Deduced amino acid sequences of Class 1 protein (Por A) from three strains of *Neisseria meningitidis*. J. Exp. Med. 171 (1990) 1871–1882.

Mullis, K.B.: The polymerase chain reaction in an anemic mode: how to avoid cold oligodeoxyribonuclear fusion. PCR Methods Applic. 1 (1991) 1–4.

Mullis, K.B. and Faloona, F.A.: Specific synthesis of DNA in vitro via a polymerase catalyzed chain reaction. Methods Enzymol. 155 (1987) 335–340.

Ni, H., Knight, A.I., Cartwright, K., Palmer, W.H. and McFadden, J.: Polymerase chain reaction for diagnosis of meningococcal meningitis. Lancet 340 (1992) 1432–1434.

Rao, V.B. and Saunders, N.B.: A rapid polymerase-chain-reaction-directed sequencing strategy using a thermostable DNA polymerase from *Thermus flavus*. Gene 113 (1992) 17–23.

Schneider, H., Hammack, C.A., Apicella, M.A. and Griffiss, J.M.: Instability of expression of lipooligosaccharides and their epitopes in *Neisseria* and *Gonorrhoeae*. Infect. Immun. 56 (1988) 942–946.

Van der Ley, P., Heckels, J.E., Virji, M., Hoogerhout, P. and Poolman, J.T.: Topology of outer membrane porins in pathogenic *Neisseria* spp. Infect. Immun. 59 (1991) 2963–2971.

Varas, F., Medrano, L., Ballester, S. and Najera, R.: Influence of PCR parameters on amplifications of HIV-1 DNA: establishment of limiting sensitivity. Biotechniques 11 (1991) 384–391.

Wedege, E., Dalseg, R., Caugant, D.A., Poolman, J.T. and Froholm, L.O.: Expression of an inaccessible P1.7 subtype epitope on meningococcal class 1 proteins. J. Med. Microbiol. 38 (1993) 23–28.

Weiss, M.S., Abele, U., Weckesser, J., Welte, W., Schiltz, E. and Schulz, G.E.: Molecular architecture and electrostatic properties of a bacterial porin. Science 254 (1991) 1627–1630.

Zollinger, W.D.: Meningococcal meningitis. In: Cryz Jr., S.J. (Ed.), Vaccines and Immunotherapy. Pergamon Press, New York, 1991, pp. 113–126.

Zollinger, W.D. and Boslego, J.: Immunologic methods for the diagnosis of infections by Gram-negative cocci. In: Rose, N.R., deMacario, E.C., Fahey, J.L., Friedman, H. and Penn, G.M. (Eds.), Manual of Clinical Laboratory Immunology. American Society for Microbiology, Washington, DC, 1992, pp. 440–451.

Zollinger, W.D., Moran, E.E., Connelly, H., Mandrell, R.E. and Brandt, B.: Monoclonal antibodies to serotype 2 and serotype 15 outer membrane proteins of *Neisseria meningitis* and their use in serotyping. Infect. Immun. 46 (1984) 260–266.

Zollinger, W.D., Boslego, J., Moran, E., Garcia, J., Cruz, C., Ruiz, S., Brandt, B., Martinez, M., Arthur, J., Underwood, P., Hankins, W., Mays, J., Gilly, J. and the Chilean National Committee for Meningococcal Disease: Meningococcal serogroup B vaccine protection trial and follow-up studies in Chile. Natl. Inst. Publ. Health Ann. (Oslo, Norway) 14 (1991) 211–212.